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13. ABSTRACT ***(Maximum 200 Words)***

Bone represents a common site for metastasis of prostate cancer cells, where the invading cells find themselves in an environment rich in factors which promote cancer growth and progression. Genetic changes occur during disease progression in bone which include both gene mutation and changes in the pattern of gene expression. These genetic alterations provide targets for new "molecular drugs" for metastatic prostate cancer. This project will investigate the role of an extracellular matrix protein, osteopontin, which is expressed by metastatic prostate cancer cells but not by normal prostate in the ability of prostate cancer cells to form metastases in bone. The functional properties of osteopontin, including interactions with the $\alpha_v\beta_3$ integrin and CD44 receptor, indicate that osteopontin may play an important role in tumor cell attachment, invasion and growth in the bone environment. A series of ribozymes which specifically cleave OPN mRNA sequences will be developed that will inhibit expression of the osteopontin gene. A series of experiments will be performed in stably transfected prostate cancer cell lines to determine if ribozyme-mediated destruction of the osteopontin gene product modulates cell phenotype. In particular we will examine effects on adhesive properties, growth in soft agar, cell growth rates, chemotaxis, and invasion. These studies will be designed to translate into improved therapies aimed at inhibiting the development of prostate cancer at secondary bony sites. They will also allow development of a prototype for molecular constructs that could be targeted for treatment of other types of cancer that can metastasize to the bones.

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FOREWORD

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Mary C Farach-Carson 11/1/99

PI - Signature Date

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INTRODUCTION:

It is clear that bone, primarily the bone in the lower spine, is a very common site for prostate cancer cells to invade and form metastases. The unique composition of the bones in some way causes the prostate cancer cells in this environment to acquire a more aggressive phenotype, including a loss of dependence on androgens for growth. Additionally, cells from bony metastases readily leave the bone in advanced disease, where in a more dangerous form they form metastases in other tissues including lung and brain. There is presently no very effective treatment for prostate cancer once it has metastasized to the bone. We propose to develop a new kind of therapy for advanced prostate cancer that has metastasized to the bone. Specifically, we plan to design a series of "molecular drugs" called ribozymes that will shut down the expression of osteopontin in transfected cells. Osteopontin turns on in prostate cancer cells when they metastasize to bone, a phenomenon which is likely associated with the change in phenotype. We will test if shutting down the expression of osteopontin reduces the ability of prostate cancer cells in bone to progress to a more aggressive phenotype. If so, we could also eventually test this approach for other types of cancer cells that also metastasize to bone such as breast cancer. Our aim is to discover why the osteopontin gene turns on in prostate cancer cells at sites of bony metastasis. We also plan to investigate how osteopontin expression associates with the changes in phenotype that have been observed. These findings could open the door to the development of other molecular ribozyme gene therapies that could be used in similar fashion.

BODY:

The research accomplishments are detailed below. To the best of our ability, we follow the organization set forth in the original statement of work. Some studies were postponed because of negative experimental findings or unanticipated technical difficulties. Other exciting new observations were made and incorporated into the experimental plan. The specific information requested is provided by task and milestone. Note that all data are consistent with the original hypothesis.

Task 1. Design and clone ribozymes with inducible promoters.

Comment: We have designed and cloned two active ribozymes. A third would not clone out for reasons that we have not determined. We have shifted from a strategy to employ an inducible promoter to one that employs a constitutively active promoter because we found the inducible promoter to be "leaky". The ribozymes that we have made are active *in vitro*, and cleave the osteopontin transcript *in vitro*. The sequences of the ribozymes are as predicted, and the constructs appear to be stable. We feel that the two ribozymes we have made and cloned are equally good candidates for later studies. **This completes task 1.**

Task 2. Transfect OPN ribozymes into prostate cancer cells.

Comment: We successfully cloned both active ribozymes into PC-3 cells and selected stable colonies expressing the ribozyme construct constitutively. The assay employed to detect the ribozyme insert and the plasmid vector was a polymerase chain reaction based assay with specific primers. The cell lines expressing the ribozymes are maintained in neomycin selection medium and appear to be completely viable. We have optimized conditions for transfection. The

aim to demonstrate inducible expression was dropped because of the leakiness of the inducible promoter in the presence of androgen used to maintain some androgen-dependent lines such as LNCaP. We felt that switching to constitutively active constructs would allow us to directly compare androgen-dependent and androgen-independent lines as stated in our original specific aims. **This completes task 2.**

MILESTONE 1: Achieved, proceeded to task 3.

Task 3. Confirm ability of ribozymes to regulate levels of OPN expression.

Comment: We had no problem growing cells in the presence of active ribozyme so long as the cells were maintained in selection medium. We have developed an RT-PCR assay to quantitate levels of OPN transcript in various cell lines, and have also used a dot blot assay and Northern blots for quantitation. There have been no problems extracting RNA containing full length transcripts encoding OPN. We have also developed assays to quantitate levels of protein expression by Western blotting using polyclonal anti-OPN antibodies made in goat. FACS analysis has been used to demonstrate cell surface expression of the OPN protein. These techniques were used to assess the levels of OPN transcript and protein in various prostate cancer cell lines, and demonstrate increased expression associated with disease progression. These data are presented in manuscript 1 included as part of this progress report (Thalmann et al, 1999). We are presently in the process of assessing the levels of OPN transcript and protein expression in the ribozyme-transfected PC-3 cells. **Task 3 is partially complete and the latter parts are still ongoing.**

MILESTONE 2: We have partially achieved milestone 2. We have delayed studies with ribozyme transfected cell lines until we have optimized the matrix influences that maximally up-regulate OPN expression (see below).

Task 4. Begin biological assays to determine the role of OPN in various assays. Compare the behavior of control and ribozyme-expressing cell lines.

Comment: We have developed reliable assays to measure the effects of OPN on cell adhesion, proliferation and survival, attachment to matrix, and focus formation in soft agar. Two very interesting and unexpected results emerged from these assays. First, we found that OPN itself stimulates a growth response in prostate cancer cells, particularly those conditioned to grow in bone matrix. As an index of growth signal, we devised an assay to measure the ability of bone matrix proteins to stimulate the development of calcium transients in PC-3 cells. These assays employed the fluorescent indicator fura-2, and clearly showed that OPN stimulated a transient that could be blocked by LM-609, an antibody that blocks the function of the integrin, $\alpha_v\beta_3$. These results were submittted and are in press, attached to this report as manuscript 2 (Lecrone et al., in press). A second very interesting finding is that the expression of OPN by prostate cancer cell lines is dependent on the matrix on which the cells are cultured. A present priority is to optimize the matrix substrate for growing PC-3 cells and maintain high OPN expression levels. We will then compare the influence of the matrix on the control and ribozyme-transfected cells. **Task 4 is ongoing and expected to occupy much of the current year of research.**

Tasks 5 & 6. Data analysis and report writing.

Comment: **These tasks are ongoing as we make progress on the biological research tasks described above.** Two manuscripts have appeared.

KEY RESEARCH ACCOMPLISHMENTS:

1) Design and cloning of two active ribozymes directed specifically toward OPN.

2) Transfection and stable expression of OPN ribozymes in PC-3 cells.

3) Development of assays for reliable detection of OPN protein and transcript.

4) Development of bioassays for adhesion, proliferation, differentiation and growth stimulation of various cell lines in culture.

5) Demonstration that OPN biosynthesis correlates with increased metastatic potential.

6) Demonstration that OPN expression is dependent upon the content of the biological matrix upon which the cells are growing.

REPORTABLE OUTCOMES:

Manuscripts.
1. Thalmann (add complete reference)
2. Lecrone (add complete reference)

Patents and Licenses.

Not applicable

Degrees obtained.

Mr. Jeff Kiefer is a full time graduate student who is seeking a Ph.D. He is expected to complete degree requirements in 2000.

Cell lines.

The ribozyme-transfected cell lines will be available at the conclusion of these studies. They are not yet completely characterized.

Databases and animal models.

Not applicable.

Funding.

We have not applied for other funding based upon this project at this time.

Training.

Dr. Wei Li, who was a research associate working in part on this project, has left the lab to pursue a residency in pathology at the University of Texas-Houston, Medical School. He will continue his clinical interest in cancer biology in this new clinical role.

CONCLUSIONS:

If shutting down osteopontin expression makes prostate cancer cells less able to live and grow in bone, we will perform animal studies to see if it will work as well in animal models of prostate cancer as it does in cultured prostate cancer cells. The animal studies will tell us if ribozyme constructs have potential as a therapy for the treatment of advanced prostate cancer that has metastasized to bone. The most direct benefit of our ideas, consistent with programmatic goals, will be to patients with advanced prostate cancer. Our hope is that ribozyme therapies will help to slow down the progress of the disease so that the quality of life can be maintained.

REFERENCES:

A comprehensive and well written review article has appeared that supports our ideas about the interactions between bone and prostate cancer cells at sites of bony metastasis. This article is referenced below:

Koeneman KS, Yeung F, Chung LW (1999) Osteomimetic properties of prostate cancer cells: a hypothesis supporting the predilection of prostate cancer metastasis and groth in the bone environment. *Prostate* 39:246-261.

APPENDICIES:

Thalmann GN, Sikes RA, Devoll RE, Kiefer JA, Markwalder R, Klima I, Farach-Carson CM, Studer UE, Chung LW (1999) Osteopontin: possible role in prostate cancer progression. *Clin. Cancer Res.* 5:2271-2277.

LeCrone V, Li W, Devoll RE, Logothetis C, Farach-Carson MC (1999) Calcium signals in prostate cancer cells: specific activation by matrix proteins associated with bony metastasis. *Cell Calcium* (in press).

Osteopontin: Possible Role in Prostate Cancer Progression[1]

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Urology Research and Gene Therapy Laboratory, Departments of Urology [G. N. T., I. K., U. E. S.] and Pathology [R. M.], University of Berne, CH-3010 Berne, Switzerland; Molecular Urology and Therapeutics, Department of Urology, University of Virginia Health Science Center, Charlottesville, Virginia 22908 [R. A. S., L. W. K. C.]; and Basic Sciences, Dental Branch, University of Texas Health Science Center, Houston, Texas 77030 [R. E. D., J. A. K., C. M. F. C.]

ABSTRACT

Human prostate cancer has the propensity to metastasize to the bone where reciprocal cellular interactions between prostate cancer and bone cells are known to occur. Osteopontin (OPN), a noncollagenous bone extracellular matrix, is a secreted adhesive glycoprotein with a functional RGD cell-binding domain that interacts with the $\alpha_v\beta_3$ cell surface integrin heterodimer. OPN has been associated with malignant transformation as well as being ligand to the CD44 receptor. Polyclonal antibodies to human OPN (hOPN) were prepared, and specificity was shown by preabsorption with recombinant hOPN. The stimulatory effect of hOPN protein and the inhibitory effect of hOPN antibody on human prostate cancer cell lines LNCaP and C4–2 were assessed by induction or inhibition of anchorage-independent growth, respectively. Expression of hOPN mRNA in prostate cancer cell lines and human prostate cancer tissue specimens were measured by mRNA blot analysis. Protein expression was assessed by immunohistochemistry in human prostate cancer specimens and by Western blot analysis in prostate cancer cell lines. hOPN stimulated anchorage-independent growth of the human prostate cancer cell lines LNCaP and C4-2 *in vitro*. Antibodies to hOPN inhibited the growth-stimulatory effect by endogenous OPN, which can be overcome by the addition of exogenous hOPN. hOPN mRNA and protein are expressed in human prostate cancer cell lines *in vitro* and in clinical human prostate cancer specimens. These findings taken together suggest that OPN may act as a paracrine and autocrine mediator of prostate cancer growth and progression.

INTRODUCTION

After an initial responsiveness to hormonal deprivation, advanced prostate cancer almost invariably relapses, and androgen-independent clones progress and metastasize nonrandomly to the axial skeleton. Experimental studies of human prostate cancer models have stressed the influence of the mesenchymal-epithelial interaction on the development of prostate cancer (1). In a series of studies, we observed that prostate and bone fibroblasts, in the appropriate host hormonal milieu, can induce the progression of prostate cancer cells from an androgen-dependent to an androgen-independent state, whereby cells with the latter phenotype express osseous metastatic potential (2, 3). It has been suggested that the development of osseous metastasis is dependent upon bone marrow and osteoblast-derived soluble growth factors that stimulate the growth of prostate cancer cells in a paracrine-mediated manner (4, 5).

Extracellular matrix has been demonstrated to play a critical role in tumor cell growth, adhesion, migration, and metastasis. Although the role of the extracellular matrix and its intracellular signaling through integrin heterodimers remain to be defined, interactions between bone matrix proteins and prostate cancer cells are likely to be the key determinants regulating prostate tumor cell behavior. The expression of bone matrix proteins is not restricted to bone tissue. One of these noncollagenous bone matrix proteins, OPN,[4] a highly posttranslationally modified glycoprotein, contains the cell attachment amino acid sequence RGD (arginine-glycine-aspartic acid), which binds to the $\alpha v\beta 3$ integrin heterodimer (6, 7). In the bone, osteoclast activity is increased as a consequence of the interaction between OPN and the osteoclast cell surface integrins. OPN is also involved in regulating bone formation and remodeling of mineralized tissues. OPN demonstrates multiple interactions with Ca^{2+}. Furthermore, OPN recruits and stimulates macrophages and lymphocytes as part of a nonspecific response to microbial infections, inhibits and disrupts the growth of calcium oxalate crystals, affects nitric oxide production, and is involved in mediating cell migration (8, 9). Recently, OPN has been reported to be a ligand of the CD44 receptor (10).

OPN has been shown to be overexpressed in human cancers (11–13), and OPN overexpression confers malignant transformation and was observed frequently in a variety of tumorigenic human cell lines (12–14). Consistent with these observations, serum levels of OPN were substantially elevated in patients with metastatic cancer (15). Ha-*ras*-transfected NIH

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[4] The abbreviations used are: OPN, osteopontin; hOPN, human OPN; pTURP, palliative transuretheral resection of the prostate; BPH, benign prostate hyperplasia; PIN, prostatic intraepithelial neoplasia; CM, conditioned medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

3T3 fibroblasts, which are tumorigenic and metastatic in contrast to untransformed fibroblasts, express increased levels of OPN. When Ha-*ras*-transfected fibroblasts were transfected with an antisense osteopontin RNA, their tumorigenic and malignant growth were reduced significantly (16). In human esophageal cancer, *ras*-regulated gene products, OPN and cathepsin L, were shown to be associated with tumor invasion and metastasis (17). We previously reported, in a preliminary finding on the potential role of bone matrix proteins such as OPN, on the progression and dissemination of prostate cancer (18).

In this communication, we have extended our previous report on the potential role of bone matrix proteins such as OPN on the progression and dissemination of prostate cancer. We have evaluated the role of OPN in human prostate cancer progression and metastasis. We have demonstrated the value of characterizing in detail the phenotypic and genotypic alterations in LNCaP lineage-related cell models of prostate cancer progression and their utility to define clinical prostate cancer. Herein we provide the experimental evidence that OPN overexpression is associated with human prostate cancer progression. We conclude that OPN may confer selective growth and malignant potential to prostate tumor cells *in situ*.

MATERIALS AND METHODS

Cell Culture. LNCaP cells, passage 29 of the original line developed by Horoszewicz *et al.* (19), were kindly supplied by Dr. Gary Miller (University of Colorado, Denver, CO). LNCaP cells (passages 37–40), C4–2 cells (passages 20–23), and NbE 1.4 cells (passages 30–35) were grown in T-medium with 5% fetal bovine serum as described previously (1, 20). DU145 and PC3 cells were purchased from American Type Culture Collection and grown in T-medium. Passages 25 to 33 of a human bone fibroblast cell line, MS, established from a patient with an osteosarcoma as described previously (4), were used in this study. The cell lines were tested and found free of *Mycoplasma*.

Purification of hOPN. hOPN was extracted and purified as published previously (21). In brief, human milk was extracted in guanidine-HCl/EDTA and was fractionated by DEAE-cellulose chromatography, eluted with 50 mM NaCl dissolved in 7 M urea (pH 4.0). A single hOPN band, migrating at M_r 60,000 and yielding a symmetrical peak on gel filtration on Sephacryl S-300, was detected.

Preparation of hOPN Antibody. hOPN purified from human milk was used as an antigen to raise hOPN polyclonal antibodies in a goat (Bethyl Laboratories, Montgomery, TX). One hundred μg of purified hOPN was injected s.c. once every 2 weeks, with the initial immunization in complete Freund's adjuvant and subsequent immunizations in incomplete Freund's adjuvant. Bleeds were taken prior to the first immunization and 7 days after each injection. Antibody titer was assessed using an ELISA. Goat anti-hOPN antibody was affinity-purified by using recombinant hOPN on an affinity column. Affinity-purified hOPN polyclonal antibodies were used for immunostaining.

Tissue Sampling. Radical prostatectomy specimens for routine histological examination were fixed in 4% paraformaldehyde and 5 mM $MgCl_2$. Six-μm, paraffin-embedded tumor sections were cut and stained with H&E. Tumors were evaluated, staged, and graded by a pathologist (R. M.). Specimens from pTURP were processed in a similar manner. Sections containing normal prostate cancer, BPH, high-grade PIN, and prostate cancer were cut and used for immunohistochemical staining. Thirty-one radical prostatectomy specimens and 8 pTURP specimens were evaluated. Tumor tissue samples for RNA blot analysis were resected and snap frozen from radical prostatectomy specimens by the pathologist. BPH and tumor tissues from TURP or pTURP were immediately snap frozen. Normal prostate tissues were obtained from organ donors according to the institutional guidelines and with approval of the University Ethical Committee. All tissues were stored at −80°C until further processing.

Immunohistochemical Staining of OPN. Immunohistochemical staining was performed on deparaffinized human prostate cancer tissue sections. Specimens were washed three times in PBS, preincubated with donkey serum 1:20 to block nonspecific activity, and then incubated with an affinity column-purified, polyclonal, goat-specific primary antibody against hOPN. The specificity of the hOPN antibody was tested by preabsorption of column chromatographically purified hOPN before immunostaining of a positive control (human bone tissue; Fig. 3, *a* and *b*). Preimmunization serum of the same animal was used as a negative control for hOPN, and human bone tissue sections served as positive control. After 3 × PBS wash preincubation with donkey serum 1:20, the tissue specimen was incubated with the secondary antibody donkey anti-goat (alkaline phosphatase) 1:50. After three PBS washes, the alkaline phosphatase substrate (Vector Red; Vector Laboratories, Inc., Burlingame, CA) was added, incubated, and washed. Slides were then stained in filtered hematoxylin, dehydrated (80% ethanol, 95% ethanol, absolute ethanol, and xylene), and mounted with Permount (Fisher Scientific Corp., Fair Lawn, NJ). Although immunostaining for OPN in cultured prostatic cancer cell lines was uniform, multifocal OPN staining was commonly observed in clinical specimens with multifocal disease. Staining for OPN was rated as follows: −, negative staining; (+), slight staining; +, positive focal staining; and ++, strong focal staining. Only positive and strong focal staining were considered as positive staining for OPN.

Soft Agar Colony Formation. To determine the ability of hOPN to stimulate colony formation in LNCaP and C4–2 cells when grown *in vitro*, TCM, a defined serum complement (Celox Co., Minnetonka, MN, see Ref. 1) and CM from NIH 3T3 fibroblasts (negative control) were used as baseline. Cells (2.5×10^3 LNCaP) and 1.0×10^3 (low density) or 5.0×10^3 (high density) C4–2 cells were trypsinized to single-cell suspensions and incubated for 4 h either with 3T3 CM, hOPN (10 μg/ml), purified hOPN antibody (dilution 1:50), preimmunization control serum, or in combination (OPN 1 μg/ml plus OPN-antibody; dilution, 1:50) after treatment with 0.02% EDTA. The concentrations for OPN (10 μg) and OPN-antibody (dilution, 1:50) were determined in preliminary experiments.

3T3 CM was prepared as follows. Cells in 70–80% confluent culture were downshifted to serum-free T-medium containing 2% TCM, a serum-free defined medium supplement only. After 48 h, CM was removed and lyophilized, and protein concentrations in the CM were determined using a protein assay (Bio-Rad Laboratories, Richmond, CA); they ranged from 70–

100% of control (T-medium and 2% TCM; 1.3 mg/ml). Samples were then reconstituted in T-medium to 2× concentration and then filtered (0.2 μm; Nalge Co., Rochester, NY).

After incubation with EDTA, $MgCl_2$ and $CaCl_2$ were supplemented, and the cells were resuspended in 0.3% agarose containing T-medium supplemented with 2% TCM and plated on a 12-well plate (Costar, Cambridge, MA) containing 0.6% agarose as a bottom layer. The cover layer, consisting of T-medium and 2% TCM, was changed weekly. Colonies larger than 0.1 mm were scored 4–6 weeks after plating.

RNA Blot Analysis. Total cellular RNA was extracted from cells (LNCaP, C4–2, DU145, PC-3, NbE1.4, and 3T3) and from snap frozen, pestle-ground human tissue samples by the RNAzolB method (Biotech Laboratories, Inc., Houston, TX.), a single step purification protocol. Thirty μg of RNA, as determined by absorbency at 260 nm, were subjected to RNA blot analysis by electrophoresis in a 0.9% agarose gel containing 2 M formaldehyde. RNAs were transferred by capillary blotting onto Zetaprobe membrane (Bio-Rad, Richmond, Calif.) using 1× TAE buffer. RNAs were cross-linked to the membranes by UV exposure using a stratalinker (Stratagene, La Jolla, Calif.) at 1500 mJ and prehybridized in hybridization buffer (10% dextran sulfate, 1% SSC, 1 M NaCl, and 20 μg/ml salmon sperm DNA from Amersham, Inc., Arlington Hts. IL). The solution hybridization was performed by incubation at 65°C overnight by exposing the membranes to a ^{32}P-labeled OPN or GAPDH cDNA (loading control) probe, having specific activity $>1 \times 10^8$ dpm/μg. After hybridization, the membranes were washed in 2× SSC at room temperature for 30 min, then washed under highly stringent conditions (twice for 30 min in 2× SSC/1% SDS, then once for 30 min in 0.5× SSC/1% SDS) at 65°C. Autoradiograms were prepared by exposing Kodak X-Omat AR films to the membrane at −80°C with intensifying screens. Autoradiograms of RNA blot analysis for OPN and GAPDH were analyzed, and OPN values were normalized to GAPDH by means of an AMBIS Molecular Dynamics Imaging System (Molecular Dynamics, Sunnyvale, CA).

Western Blot Analysis. Recombinant hOPN (20 μg) and cell lysate samples (20 μg) were run on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose paper by electrophoresis. After blocking with PBST (PBS with 0.1% Tween 20), the blots were incubated with the indicated primary antibody (hOPN-AB) at a dilution of 1:250 at 4°C for 12 h. The blots were then washed three times with PBST buffer and incubated with the secondary streptavidin peroxidase-conjugated antibody for 1 h at room temperature. After being washed three times, the protein bands were detected by enhanced chemiluminescence (Amersham, Inc.).

Intracellular Quantification of OPN by Flow Cytometry. Cells (1×10^6) were harvested and washed with 1× PBS and then fixed with 2% paraformaldeyhe at 4°C for 30 min. After fixation, cells were washed twice in ice cold permeabilization buffer [PB: PBS (pH 7.4), 0.1% saponin, and 0.1% BSA] and incubated with goat anti-OPN in PB (1:250) for 30 min on ice. Negative control antibody consisted of normal goat serum (Sigma) used at a dilution of 1:250. Cells were collected by centrifugation, washed twice with cold PB buffer, and incubated with secondary rabbit anti-goat FITC (1:500) in PB for 30 min on ice. Cells were collected by centrifugation, washed twice with cold PB buffer, resuspended in cold PBS, and analyzed on a FACScan flow cytometer (Becton Dickson). Results are expressed as a ratio of mean channel fluorescence of test antibody to that of negative control serum. Mean channel fluorescence of the control antibody is defined as 1.0. Upon the addition of OPN antibody, the mean channel fluorescence shifted to the right, an indication of increasing fluorescence intensity. The ratios were calculated and represent a semiquantitative estimation of OPN protein content in the cells.

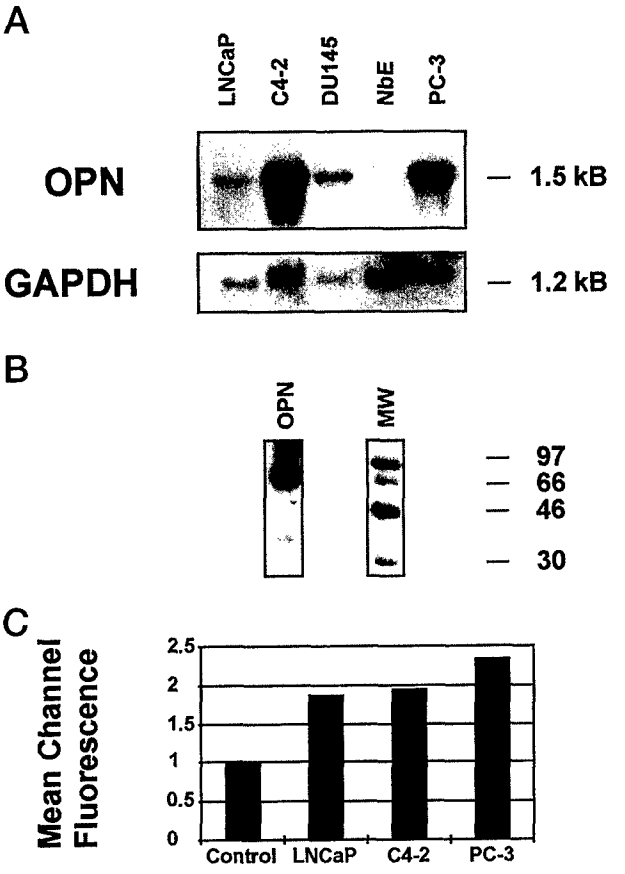

Fig. 1 OPN expression by different prostate cancer cell lines. *a*, RNA blot analysis of OPN expression. GAPDH serves as a loading control. Cell lines denoted LNCaP, C4–2, DU 145, NbE, and PC-3. *b*, Western blot analysis of hOPN protein on 10% SDS-polyacrylamide gel with goat anti-hOPN antibody at 1:250 dilution. *c*, intracellular quantification of OPN by flow cytometry of different prostate cancer cell lines with hOPN antibody. Cell lines shown: LNCaP, C4–2, and PC-3. Results are expressed as a ratio of mean channel fluorescence of test antibody to that of negative control serum.

Statistical Analysis. For statistical analysis of soft agar colony-forming activity, a one-sided Student's *t* test with unequal variances was used (SPSS).

RESULTS

RNA blot analysis of several human prostate cancer epithelial cell lines (Fig. 1a) revealed an elevated expression of OPN mRNA in androgen-independent and tumorigenic cell lines C4–2, DU145, and PC-3 and to a lesser extent in the and the androgen-dependent LNCaP cell line (Fig. 1*a*). The normal

rat prostatic epithelial cell line NbE-1.4, derived from Nb rats, did not express OPN mRNA. By Western blot analysis, the antibody against hOPN (hOPN-AB) detected hOPN protein at M_r 60,000 (Fig. 1*b*). Fluorescence-activated cell sorter analysis with the hOPN antibody demonstrated that the cell lines LNCaP, C4–2, DU145, and PC3 express OPN protein on their surface (Fig. 1*c*). Western blot analysis confirmed these findings (data not shown).

Because conditioned media collected from bone fibroblasts stimulated human prostate cancer growth *in vivo* (22), we examined further whether osteopontin may support the anchorage-independent growth (soft agar colony formation of prostate epithelial cells) *in vitro* because this activity correlates closely with tumorigenicity of target epithelial cells *in vivo* (23). hOPN (10 μg/ml) stimulated the anchorage-independent growth of androgen-dependent LNCaP cells in a soft agar colony formation assay ($P = 0.013$; Fig. 2*a*). hOPN antibody did not significantly alter soft agar colony-forming activity. hOPN added in excess (200 μg/ml) could overcome hOPN antibody inhibition. The addition of CM derived from NIH3T3 cells did not significantly stimulate the anchorage-independent growth of the LNCaP cells (baseline level was defined by the addition of a serum-free supplement, 2% TCM).

Similar findings were demonstrated in the androgen-independent C4–2 cells (Fig. 2*b*) at low density (1.0×10^3 cells); hOPN (10 μg/ml) induced anchorage-independent growth ($P < 0.01$). Unlike LNCaP cells, hOPN antibody alone decreased the baseline number of soft agar colonies formed by C4–2 cells below baseline ($P = 0.038$), and again this inhibition could be overcome by exogenously added hOPN (200 μg/ml). At a higher density (5.0×10^3 cells), androgen-independent C4–2 cells demonstrated an elevated intrinsic anchorage-independent growth at baseline. Under these autocrine stimulatory conditions, hOPN did not significantly stimulate anchorage-independent growth, but the addition of hOPN antibody inhibited this intrinsic androgen-independent growth (data not shown). Again, the hOPN antibody inhibition could be overcome by exogenous hOPN (data not shown). The preimmune serum of the same animal did not inhibit anchorage-independent growth in preliminary experiments (data not shown).

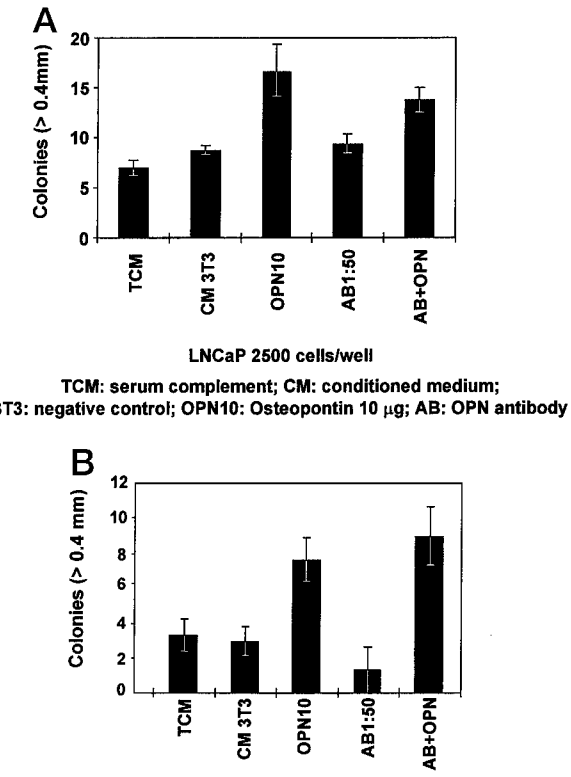

Fig. 2 Anchorage-independent growth of human prostate cancer cell lines LNCaP and C4–2. *a,* anchorage-independent growth by androgen-dependent LNCaP. Stimulation of LNCaP soft agar colony formation activity by 10 μg of OPN ($P = 0.013$). *b,* anchorage-independent growth of androgen-independent C4–2 cells. Stimulation of C4–2 soft agar colony formation activity by 10 μg of OPN ($P < 0.01$). hOPN antibody (hOPN-AB) in a 1:50 dilution significantly decreases anchorage-independent growth below baseline ($P = 0.038$). hOPN AB inhibition can be overcome by exogenous hOPN. *TCM,* defined serum complement; *CM,* conditioned medium; *3T3,* NIH 3T3 fibroblasts; *OPN10,* hOPN 10 μg; *AB,* hOPN antibody.

To demonstrate the presence of OPN in human tissues, we conducted immunohistochemical analysis of clinical specimens of prostate cancers obtained from radical prostectomy and transurethral resections. As a positive control and to demonstrate the specificity of the hOPN antibody, we stained normal human bone for OPN (Fig. 3*a*), which exhibited distinct staining of the reversal lines and osteocytes. Preabsorption of the hOPN antibody with affinity-purified hOPN abolished the immunostaining (Fig. 3*b*). Immunohistochemical staining of pathologically normal human prostate showed no immunostaining or only slight staining of the luminal surface of atrophic glands, a result in agreement with the lack of OPN in a normal rat prostatic epithelial cell line, NbE-1.4. Prostate cancer showed low staining intensity in low Gleason grade prostate cancer (Fig. 3*c*) and more intense immunostaining in high Gleason grade (Fig. 3*d*) and androgen-independent prostate cancer (Fig. 3*e*). BPH did not stain positive for hOPN antibody (Fig. 3*f*), which is in contrast to the mRNA blot analysis. BPH often has an inflammatory component that is associated with macrophages, which stain strongly positive for OPN, as shown in this TURP specimen (Fig. 3*g*). The results of the 39 prostate tumors are summarized in Table 1.

Fifty-eight % (18 of 31) of the peripheral zone prostate cancers from radical prostatectomy specimens with a Gleason score 6 and higher stained positive for OPN, with a more intense staining in higher grade tumors. Three of four (75%) transitional zone cancers stained positively for OPN, and two of eight (25%) of the prostate cancer specimens obtained from palliative transurethral resection stained positively for OPN. High-grade PIN stained positive in 4 of 12 (33%) cases. All of the patients ($n = 6$) with Gleason score 9 prostate cancer showed positive staining for OPN, as did 50, 58, and 67% of patients with Gleason scores 8, 7, and 6, respectively. Fig. 3*h* shows representative stainings of such OPN-positive tumors.

RNA blot analysis of tumor tissues demonstrated, with few exceptions, that OPN mRNA is widely expressed by prostate

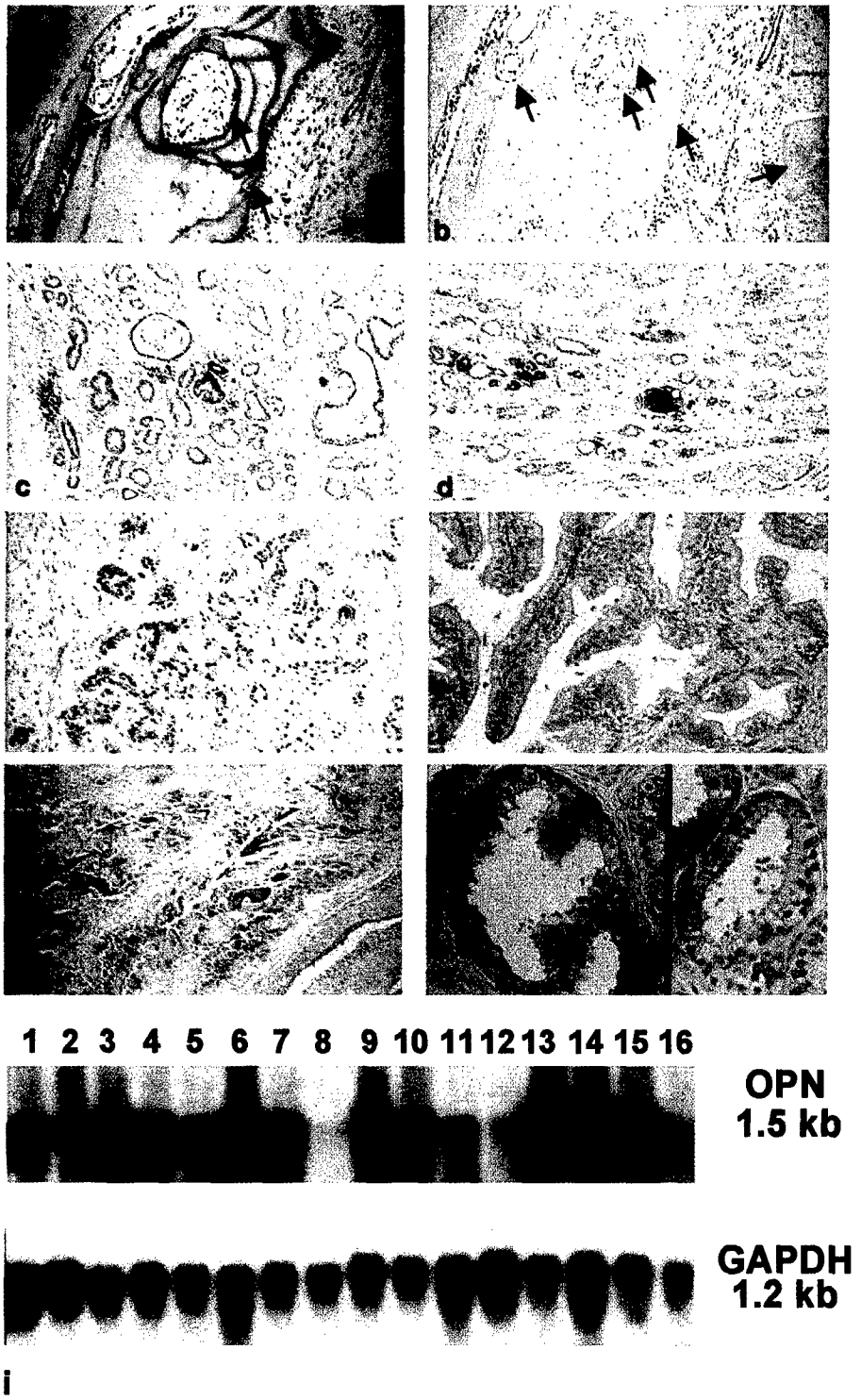

Fig. 3 OPN expression by human prostate cancer specimens and by human bone (control). *a,* immunohistochemical staining of human bone with hOPN antibody (hOPN-AB). *Arrows,* positive staining of reversal lines. *b,* immunohistochemical staining of human bone after preabsorption of the hOPN-AB with recombinant hOPN. Lack of staining indicating specificity of the hOPN-AB. *c–e,* immunohistochemical staining of prostate cancer: *c,* focal staining in low Gleason grade tumor (×10); *d,* diffuse staining in high Gleason grade tumor (×10); *e,* diffuse staining in hormone-refractory prostate cancer (×10); *f,* negative staining of BPH tissue (×10); *g,* macrophages staining positive in a TURP specimen; *h,* ×40 of prostate cancer staining; *i,* RNA blot analysis for OPN expression in human prostate cancer tissues derived from patients undergoing radical prostatectomy or palliative transurethral resection of the prostate. GAPDH serves as loading control. *Lane 1,* C4–2 mRNA (positive control); *Lane 2,* NIH3T3 mRNA (negative control); *Lanes 3–5* and *16,* BPH; *Lanes 6–12,* primary prostate cancers without metastasis; *Lanes 13–15,* primary metastatic prostate cancer.

cancer specimens (Fig. 3*i*). Interestingly, tumor specimens derived from patients with distant metastasis (*e.g.,* lymph node involvement and bone metastasis) showed higher levels of OPN mRNA expression. BPH specimens demonstrated OPN mRNA expression at a lower level.

DISCUSSION

OPN, a noncollagenous bone matrix protein, was found to be prevalently expressed by human prostate cancer cell lines and clinical prostate cancer specimens at the levels of both mRNA and protein. OPN also stimulates anchorage-independent growth

Table 1 Immunohistochemical staining of human prostate cancer specimens with the OPN antibody by tumor site

Tumor site	n[a]	Positive[b] (%)
Peripheral zone cancers	31	18/31 (58)
Transition zone cancers	4	3/4 (75)
High-grade PIN	12	4/12 (33)
Palliative transurethral resections	8	2/8 (25)

[a] *n*, number of specimens.
[b] Focal staining that is positive (+) or strongly positive (++).

of both androgen-dependent LNCaP and androgen-independent C4–2 human prostate cancer cells *in vitro*. On the basis of the data from blocking hOPN activity by hOPN antibody, it is conceivable that OPN may be one of the active extracellular matrix components that contribute to anchorage-independent growth of human prostate cancer cells *in vitro*. Although presently it is not known how OPN may induce anchorage-independent growth of prostate cancer cells, interaction between OPN and its cell surface receptor $\alpha v\beta 3$ integrin isotype in cancer cells has been observed to transduce cell-matrix signaling that culminates in increased cell motility, invasion, angiogenesis, and the ability to proliferate under anchorage-independent conditions (24). In addition, the role of CD44 receptors in the mediation of metastatic processes via OPN remains to be determined (25).

Prostate cancer cells commonly adhere to and proliferate in bone. These events are characterized by the induction of an osteoblastic reaction in the axial skeleton. The mechanisms underlying this preferential homing to the bone are unclear. Experimental data support several proposed mechanisms. For example, bone-derived growth factors have been identified and appear to play a key role in augumenting prostate cancer cell proliferation in the skeleton (1, 5). OPN has been shown in a previous study to recruit a quiescent human prostate epithelial cell population into proliferative phase (26). OPN could also alter the tumor microenvironment and for example alter the local nitric oxide production by macrophages to favor tumor growth and androgen-independent progression. These results are expanded in the present study, where OPN may be the major soluble factor secreted by osteoblasts as well as prostate cancer cells that accounts for stimulated anchorage-independent growth of prostate cancer cells *in vitro*. In addition to OPN overexpression by prostate cancer cells, several other skeletal and matrix-associated proteins, such as bone morphogenetic proteins 2, 3, and 4 (27) and bone sialoprotein (28), are also expressed by prostate cancer cells. It is conceivable that overexpressed skeletal and bone matrix-associated proteins may allow prostate cancer cells to gain adhesive, growth, and survival advantages that eventually progress to become androgen independent and metastatic with a predilection for spreading to the bone. In support of this concept, we observed for the first time that clinical prostate tumor specimens of high Gleason grade or metastatic specimens expressed high levels of OPN. Our results also support the notion that cultured prostate cancer cell lines with cell lineage relationship, such as LNCaP and C4–2 cells, provide efficient and reliable means to detect biomarkers, such as OPN, that may be associated with human prostate cancer progression.

In summary, OPN mRNA and protein are expressed in prostate cancer cell lines and human prostate cancer specimens. The malignant and metastatic phenotype seems to be correlated with elevated OPN expression. These findings suggest a paracrine and autocrine role of OPN in prostate cancer growth, invasion, and metastasis.

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Calcium Signals in Prostate Cancer Cells: Specific Activation by Matrix Proteins Associated with Bony Metastasis

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Running title: Calcium Signals in Prostate Cancer Cells

Key Words: Extracellular matrix, Osteopontin, Thrombin, Cancer, Calcium signals

Abstract: Cancer of the prostate commonly metastasizes to bony sites where cells acquire an aggressive, rapidly proliferating, androgen-independent phenotype. The interaction between bone and prostate thus becomes a key factor in disease progression. Fluctuations in intracellular ionized Ca^{2+} $[Ca^{2+}]_i$ are rapid, regulated signal transduction events often associated with cell proliferation. Hence, Ca^{2+} signals provide a convenient measure of early events in cancer cell growth. This study developed single cell fluorescent imaging techniques to visualize Ca^{2+} signals in fura-2 loaded prostatic cancer cell lines of various metastatic phenotypes. Solubilized bone fractions containing extracellular matrix and associated proteins were tested for the ability to trigger Ca^{2+} signals in prostate cancer cell lines. Fractions representing the complete repertoire of non-collagenous proteins present in mineralized bone were tested. Results demonstrated that two bone fractions termed D3b and D4a triggered Ca^{2+} signals in prostate cancer cells derived from bone (PC-3), but not brain (DU-145) metastases of prostate cancer. Lymph node derived LNCaP cells also did not produce a Ca^{2+} signal in response to addition of soluble bone matrix. No other bone fractions produced a Ca^{2+} signal in PC-3 cells. It is of interest that bone fractions D3b and D4a contain a number of non-collagenous matrix proteins including osteonectin (SPARC) and osteopontin (OPN), as well as prothrombin. Moreover, antibody LM609 that recognizes the $\alpha_v\beta_3$ integrin, blocks the ability of OPN to trigger a Ca^{2+} transient in PC-3 cells. These studies support a conclusion that bone matrix proteins play a role in the growth and progression of metastatic prostate cancer, and that prior growth in bone may be associated with development of a bone matrix-responsive phenotype.

Introduction:

Prostate cancer is now the most common type of male malignancy in the United States and the second leading cause of male cancer-related deaths. The number of newly diagnosed cases has doubled in the last decade, and the associated increase in mortality represents a major health issue for males in the United States (1,2). Carcinoma of the prostate is rarely recognized before the age of fifty, and the incidence of the disease increases with subsequent decades (2). Disease progression follows one of several pathways: peri-prostatic and peri-vesicular penetration and perforation of the prostatic capsule and invasion along the peri-neural spaces; metastasis to pelvic lymph nodes; and metastasis to bones of the pelvis, lumbar spine, thorax and other distal bony sites; finally, metastasis to soft tissues including the brain. Involvement of the axial skeleton is common, and at autopsy, 80% of those with prostatic adenocarcinoma have skeletal metastases (1,3-5). Because prostate cancer predictably metastasizes to bone, it provides a logical system in which to study the role of bone extracellular matrix proteins in cancer progression and a target for therapeutic intervention.

The composition and architecture of bone provides a uniquely rich environment to support the proliferation of cancerous metastases. The molecular structure of bone consists of fibrils of type I collagen, which constitute approximately 90% of the organic matrix. Collagen fibrils may be found in lamellar bone in an ordered array, or in woven bone (6). Randomly ordered fibrils of collagen, similar to those in woven bone, appear during the osteoblastic response that characterizes prostate cancer invasion of bone. In addition to collagen, bone consists of noncollagenous matrix proteins, serum-derived globular proteins, and sequestered cytokines and growth factors (7). Some of the non-

collagenous matrix proteins include osteonectin (SPARC), osteopontin (OPN), bone sialoprotein, and the small bone proteoglycans biglycan and decorin (7). The identity of the active factors in bone matrix that contribute to accelerated growth and increased metastatic potential of prostate cancer cells has been a recent focus of our group (8).

Ca^{2+} signals provide a convenient measure of the ability of a cell to respond to an extracellular stimulus such as provided by a growth factor (9-11). Fluorescent Ca^{2+}-sensitive dyes such as fura-2 have easily measurable optical responsiveness to Ca^{2+} binding, and rapid kinetics which allow measurement of intracellular Ca^{2+} concentrations in real time. In this study, we measured Ca^{2+} signals in several prostate cancer cell lines in response to soluble bone matrix proteins. Cell lines included those derived from lymph node, brain and bone metastases.

Methods:

Materials: Coverslip dishes were obtained from MatTek Corp. (Ashland, MA). Fura-2/AM, the acetoxymethyl ester of the Ca^{2+} -sensitive dye, fura-2, was purchased from Molecular Probes, Inc. (Eugene, OR). Thapsigargin was from Calbiochem Corp. (La Jolla, CA). Thrombin (rat plasma), prothrombin (rat plasma), and adenosine 5'-phosphate (ATP) were purchased from Sigma Chemical Co. (St. Louis, MO). Osteonectin (SPARC) and SPARC polyclonal antibody were provided by Dr. Helene Sage, and α_2HS serum glycoprotein was purified in our laboratory as described previously (12). PC-3 and DU-145 cell lines were obtained from the American Type Tissue Culture Collection (Rockville, MD). Antibody LM609 was provided by Chemicon International Inc. (Temecula, CA).

Fractionation and Characterization of Bone Matrix Proteins: Rat long bones were dissected, crushed, demineralized, and extracted as described previously (13). Non-collagenous proteins were separated into a series of protein fractions using a combination of gel filtration and anion exchange chromatography as described previously (14). The final collection of protein fractions used in this study included fractions referred to as ES-2 (small molecular weight bone proteins from the initial gel filtration step) and a series of fractions in order of increasing anionic charge D1, D2, D3a,b, D4a,b, and D5. A general description of the major components of these fractions was described previously and is shown compiled with current data in table I (14). Protein sequence analysis (amino terminal) was routinely performed on a fee for service basis at the Baylor College of Medicine (Houston, Texas) core facility following sodium dodecyl sulfate polyacrylamide

gel electrophoresis (SDS-PAGE) or chromatographic separation as described previously (15).

Cell Culture: Two human prostate cancer cell lines of different metastatic origin were used for this study. PC-3 cells were derived from a patient with bone metastasis, whereas DU-145 cells were derived from a patient with brain metastasis (16,17). Both cell lines were cultured in Dulbecco's Modified Eagle's Medium-Ham's F12 (1:1; DMEM-F12) medium containing 10% fetal bovine serum (FBS). The well characterized, androgen-regulated cell line LNCaP was also studied, and cultured under well-defined conditions (18). Two recently isolated cell lines also were obtained from Dr. Nora Navone (University of Texas M.D. Anderson Cancer Center) that represent an intermediate cell type between the androgen-independent PC-3 and DU-145 cells and androgen-regulated LNCaP cells. These cells, A10(PCA2a) and A11(PCA2b) were isolated from bone metastases, but remain fairly slow growing and androgen-responsive (19). Cells were plated onto coverslip dishes in DMEM-F12 medium containing 10% FBS two days prior to the day of the experiment. All cells were subconfluent at the time of the experiments.

Intracellular Ca^{2+} Measurements: A single cell Ca^{2+} imaging system (Intracellular Imaging, Inc., Cincinnati, OH) was used to perform intracellular Ca^{2+} measurements as described previously (20). After removing the medium from the dishes, cells were rinsed with supplemented Hank's Balanced Salt Solution (HBSS), [140 mM NaCl, 4.2 mM KCl, 0.5 mM NaH_2PO_4, 0.4 mM Na_2HPO_4, 0.4 mM $MgSO_4$, 0.3 mM $MgCl_2$, 1 mM $CaCl_2$, 6 mM glucose, 0.1 % FBS, and 20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), pH 7.4). Rinsed cells were loaded with 3 μM fura-2/AM in supplemented HBSS for 30 min at 37° C. The conditions were chosen to avoid probe

compartmentalization and to maximize cytoplasmic dye localization. The loaded cells were incubated for another 15 min with supplemented HBSS alone to allow complete de-esterification of the fluorescent probe. Fura-2 fluorescence was visualized with a Nikon inverted microscope using a Nikon 40X fluor objective. The cells were illuminated with a Xenon lamp equipped with quartz collector lenses. A shutter and filter changer with two different interference filters (340 and 380 nm) were computer controlled. Emitted light was passed througha 430 nm dichroic mirror, filtered at 510 nm, and imaged with an integrating CCD video camera. Four to eight cells were selected for measurement within each visual field. Consecutive frames obtained at 340 and 380 nm were used to obtain a fluorescence ratio (F_{340}/F_{380}), and $[Ca^{2+}]_i$ in each well was calculated from F_{340}/F_{380} by comparison with fura-2 free acid standards. Individual Ca^{2+} traces shown in the figures are population means derived from simultaneous recording of $[Ca^{2+}]_i$ in the 4-8 single cells in a microscopic field. Each experiment was repeated at least three times, and the figures were constructed from representative experiments.

Results:

Ability of Bone Fractions to Stimulate Ca^{2+} Influx. Table 1 provides the nomenclature used and the known composition of the bone fractions employed in this study. In total, the fractions studied include all non-collagenous proteins present in demineralized bone matrix, as well as some soluble collagen type I present in fractions ES1 (the parent fraction) and fraction D1 (the least acidic fraction eluting first from the DEAE column). They are free of small molecules removed during previous size exclusion chromatography (13). While many of the components and their elution positions on DEAE were known prior to this study, the identity of the three components of the fraction designated D3b was previously unknown (see below in Results). It is of note that fraction D4a consists primarily of OPN with properties previously described (13), although a more highly phosphorylated form (21) is present in fraction D4b along with bone sialoprotein. In keeping with recently adopted nomenclature in our laboratory, the form of OPN present in fraction D4a, that represents the major OPN found in bone matrix, is called OPN2, and is the isoform produced by bone cells treated with $1,25(OH)_2D_3$ (21) and interestingly by transformed epithelial cells (22).

In the first series of experiments we performed, soluble bone fractions at various concentrations ranging from 1-10 μg/ml, except for ES1 which was also tested at 100 μg/ml, were added to low density cultures of DU-145 or PC-3 cells loaded with fura-2 on coverslips. Using the Ca^{2+} imaging instrument, the ability of the bone proteins to trigger a measurable Ca^{2+} transient at any concentration was assessed. Almost all bone fractions failed to raise intracellular Ca^{2+} levels beyond the resting levels (near 50 nM) at any tested concentration, with the exception of two. As shown in figure 1, addition of protein

fractions D3b and D4a rapidly but transiently increased intracellular Ca^{2+} concentration to levels approaching 200 nM in PC-3 (panels A and C), but not DU-145 (panels B and D), cells. In all cells tested, subsequent addition of thapsigargin triggered a large Ca^{2+} transient, indicating that all cells were capable of storing and releasing Ca^{2+} from intracellular stores (data not shown). Fraction D4a was known to consist primarily of OPN, but the identity of the active component(s) in fraction D3b was unknown. We next tested the ability of fractions D4a and D3b to stimulate Ca^{2+} transients in three androgen-responsive cell lines including LNCaP (from lymph node) and lines A10 (PCA2a) and A11 (PCA2b) (from bone). Neither fraction elicited a positive result in any of these steroid-responsive lines, regardless of origin. Thus the ability of the bone fractions to trigger Ca^{2+} transients, and potentially growth responses, was unique to the bone-adapted, androgen-independent PC-3 cell line.

Extracellular ATP Stimulates Ca^{2+} Oscillations in Both PC-3 and DU-145 Cells. Extracellular ATP is known to stimulate Ca^{2+} responses in some cell types (10,23,24). Because prostate cancer cells are often known to display a neurosecretory phenotype, evidenced by secretion of molecules including chromagranin A, we tested the ability of ATP to stimulate Ca^{2+} transients in prostate cancer cell lines including PC-3, DU-145, LNCaP, A10 and A11 (table 2). As shown in figure 2, addition of extracellular ATP (0.5 μM) produced Ca^{2+} oscillations in both PC-3 and DU-145 cells, but not in the three androgen-regulated cell lines. The association of these oscillations with secretion has not yet been investigated.

Composition of Active Bone Protein Fraction D3b. Figure 3 shows the separation of active fraction D3b into six distinct protein components by SDS-PAGE. This has now

also been accomplished by fast liquid chromatography on a commercial anion exchange column (data not shown). Subjection of proteins electroeluted from preparative gels, or after charge-based chromatographic separation, to amino terminal sequencing demonstrated the identities of some of these proteins. Band 1 is a low abundance band of approximately 80 kDa whose amino terminus was apparently blocked, preventing sequence analysis. Band 2 was identified as prothrombin. Band 3 represents a major component whose N-terminus was also blocked. Band 4 was identified as α_2HS-serum glycoprotein that is greatly enriched in mineralized bone (12). Band 4.5 (so labeled because we did not detect it immediately due to low abundance levels) corresponds to the bone protein known as osteonectin, or SPARC (25). Band 5 is a 35 or so kDa protein that is also blocked at the N-terminal. It should be noted that non-collagenous bone proteins are commonly blocked artifactually at the N-terminal during their purification from bone, which requires the strong chaotropic agent, guanidinium chloride (William T. Butler, personal communication). This finding does not necessarily mean that these proteins are blocked *in vivo*.

Ability of Prothrombin and Thrombin to Trigger Ca^{2+} Transients in PC-3 Cells. The finding of prothrombin in active fraction D3b led us to test the ability of commercially purchased prothrombin and thrombin to stimulate development of Ca^{2+} transients in PC-3 cells. At concentrations between 2 and 5 units/ml, both prothrombin (figure 4A) and thrombin (figure 4B) stimulated development of a Ca^{2+} transient. Onset of the transient after prothrombin addition was immediate, and consistently larger in magnitude for prothrombin relative to thrombin. A time lag was consistently noted after thrombin addition, and the observed transient was of shorter duration than seen with prothrombin.

This pattern was not changed by increasing the thrombin concentration (data not shown), and may be attributed to auto-proteolysis and inactivation. As shown in the pseudo-color time lapse micrographs in figure 5, the Ca^{2+} signal induced by prothrombin involved the entire cell cytoplasm. The response to thrombin and prothrombin was restricted to the PC-3 cells, and was not seen in any of the other cell lines that we tested (table 2).

Activity of other Bone Proteins in Fraction D3b. As shown in table 2, we also tested the ability of osteonectin, or SPARC, that we found in fraction D3b to trigger Ca^{2+} transients in all five cell lines. Only the PC-3 cells demonstrated a transient in response to addition of SPARC (data not shown). This transient was of lower magnitude than obtained for either D3b, D4a, thrombin or prothrombin. Additionally, antibodies to SPARC did not block the transient induced by fraction D3b. The last identified protein component in fraction D3b is α_2HS-serum glycoprotein, a liver protein enriched in bone. This protein was not able to induce a Ca^{2+} transient in any cell line that we tested, including the PC-3 cells (data not shown). It thus appears that of the protein components in bone fraction D3b known to date, prothrombin is most likely to contribute to the development of the Ca^{2+} signal.

Involvement of the Integrin $\alpha_v\beta_3$ in Development of the OPN-Induced Ca^{2+} Transient. For these studies, we used highly purified OPN that was purified from cultured osteosarcoma cells treated with $1,25(OH)_2D_3$ as described (21). This OPN isoform that we call OPN2 (21) is typical of that secreted by transformed epithelia (22), and is underphosphorylated relative to some OPN forms produced in bone. As shown in figure 6, OPN2 (10 μg/ml) produced a Ca^{2+} transient (figure 6A) in PC-3 cells that was completely abolished (figure 6B) by pre-treatment with the $\alpha_v\beta_3$ blocking antibody LM609 (26). Non-immune

antibody controls had no effect (data not shown). We consistently noted lower responses to OPN isolated from cell cultures relative to OPN isolated from intact bone (compare figure 1, panel C to figure 6, panel A). The reason(s) for this are presently unknown, but may be due to differences post-translational modifications that affect OPN function.

Discussion: This line of experimentation was undertaken to assess the role of individual bone matrix proteins in the development of Ca^{2+} signals in prostate cancer cell lines of various metastatic potential. Both androgen-regulated (LNCaP, A10, A11) and androgen-independent (PC-3, DU-145) cell lines were studied. It is known that Ca^{2+} signals often provide an early measure of the ability of a cell to respond to a growth stimulus (9-11). No previous study of this nature has been reported, although the ability of bone proteins to stimulate adhesion and/or growth of prostate cancer cells is commonly accepted (27,28). The exact identities of the active, growth-inducing components present in bone matrix remains unknown.

Our approach was to systematically test the ability of bone fractions representing the complete repertoire of non-collagenous proteins present in bone matrix to stimulate development of a Ca^{2+} transients measured by fura-2 fluorescence. Of interest, we found that the vast majority of proteins in bone had no effect whatsoever on intracellular Ca^{2+} levels in any cell line. In contrast, two bone protein fractions (D3b and D4a) consistently triggered the development of a Ca^{2+} transient in PC-3 cells. This response was unique to the PC-3 cells, and did not occur in either the DU-145 cells nor the three androgen-regulated cell lines. The selectivity of this response to the PC-3 cells is of interest because of the five cell lines tested, the PC-3 cell uniquely represents a highly metastatic, androgen-independent, prostate cancer cell line derived from a human bone metastasis (16). The DU-145 cell, while also androgen-independent, was derived from a brain metastasis, and does not, therefore, have a history of prior growth adaptation in bone (16). The LNCaP cell represents an androgen-regulated, less aggressive non-bony tumor (17). The two new cell lines A10 and A11, while both derived from bone metastases of

prostate cancer, are slow-growing and androgen-regulated (19). They therefore may not yet have developed long-term adaptation to growth in bone matrix, and in comparison to the PC-3 represent an earlier stage of disease progression. These findings suggest that the responses to bone matrix growth signals such as Ca^{2+} transients are likely to represent an adaptive response to long term growth in the bone environment, such as occurs during transformation to an androgen-independent growth state.

D3b represents a heretofore fairly uncharacterized fraction of non-collagenous bone matrix. We were, however, not completely surprised to find that this fraction stimulated the development of a Ca^{2+} transient in PC-3 cells, because previous studies in our laboratory had shown that this fraction also stimulated growth of prostatic cells in soft agar (8). Analysis of the components of bone fraction D3b revealed several surprises, including the presence of prothrombin. This finding was of particular interest because of the growing body of literature demonstrating thrombin receptor (over)expression in malignant cells (29,30), a phenomenon that may be associated with expression of plasminogen activator (30). Combined with observations that conversion of prothrombin to thrombin can actually occur on the surfaces of cancer cells (31), it is intriguing to speculate that prostate cancer cell activation by bone matrix may involve the thrombin receptor. It is likely in this case that elevations in intracellular Ca^{2+} provide early signals that may accompany activation of growth pathways. Current studies in our laboratory will further investigate this hypothesis.

Other components in bone fraction D3b represent known bone matrix proteins including osteonectin (SPARC) and α_2HS-serum glycoprotein (7). Added in purified form, α_2HS-serum glycoprotein does not produce a Ca^{2+} transient, so it is unlikely to be

the active component in D3b. Osteonectin (SPARC) does produce a moderate transient in PC-3 cells, but one that is small relative to that produced by prothrombin. We cannot rule out the possibility that SPARC, or fragments of SPARC, contributes to Ca^{2+} signals in bone-acclimated prostate cancer cells such as the PC-3 cells. Testing of the remaining protein components of D3b will require purification of large amounts of protein, proteolytic cleavage, and internal sequencing to determine their identities. These studies are planned.

The finding that protein fraction D4a, which consists almost exclusively of OPN in a low phosphorylated form (21) called OPN2, readily triggers a Ca^{2+} transient was exciting. Previous studies in our laboratory (Thalmann, Chung, Devoll, and Farach-Carson, unpublished) and others (32) have shown that this protein and its mRNA are often up-regulated by transformed epithelial cells including prostate. The more highly phosphorylated form (OPN1) appears to be less active in this regard since fraction D4b, which contains OPN1 and bone sialoprotein, did not readily stimulate a Ca^{2+} transient in PC-3 cells. We investigated this further by purifying pure OPN1 and OPN2 from ROS 17/2.8 cells, under conditions where no protein denaturation occurred. As shown in the results, OPN2 triggered a Ca^{2+} transient in PC-3 cells that was concentration-dependent and maximum at 5-10μg/ml. Because OPN is known to serve as a ligand to the integrin $\alpha_v\beta_3$ receptor, where it can activate a variety of signaling pathways (33), we tested the involvement of this receptor in PC-3 signaling induced by OPN2. The blocking antibody LM609 (26) was utilized in these studies. When PC-3 cells were pretreated with LM609, the Ca^{2+} transient induced by addition of OPN2 was completely abolished. This is most readily interpreted to mean that OPN2-induced activation of the $\alpha_v\beta_3$ receptor is an

essential step in development of a Ca^{2+} signal in PC-3 cells in response to at least some bone matrix proteins. The relationship between this activation and growth in bone matrix is a subject for further investigation. It is also of note that OPN is a substrate for thrombin cleavage, a finding that has been speculated to have an important physiological role (34).

In summary, the collective findings reported in this manuscript support the conclusion that a subset of bone matrix proteins can trigger Ca^{2+} signals in bone-adapted cancer cells of prostatic origin. These Ca^{2+} signals are likely to be associated with growth of these metastatic cells in bone. Our studies furthermore suggest that prior growth in bone may be associated with disease progression as reflected by the acquisition of a bone-matrix responsive phenotype.

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Tables:

Table 1. Designation and known composition of non-collagenous bone fractions tested in Ca^{2+} imaging experiments.

Common Fraction Name[a]	Known composition
ES1	Large non-collagenous proteins; parent fraction of D-subfractions
ES2	Small non-collagenous proteins, primarily osteocalcin (OCN)
D1	Some soluble collagen fragments; large non-acidic serum proteins
D2	α_2HS-serum glycoprotein
D3a	Unknown
D3b	Osteonectin (SPARC); vitronectin, (pro)thrombin
D4a	Osteopontin (OPN)
D4b	Bone Sialoprotein (BSP); High phosphate-containing OPN; Dentin Sialoprotein
D5	Small bone proteoglycans: decorin and biglycan; bone acidic glycoprotein-75

[a] Demineralized rat bone extracts were resolved into two major fractions termed ES1, ES2 by gel permeation column chromatography as previously described (14). The ES1 fraction was further fractionated using a cation exchange column eluted with a linear gradient of NaCl, which produced the subfractions designated D1 to D5.

Table 2. Summary of Ca^{2+} Signaling Responses in Prostatic Cancer Cell Lines[a]

	D3b	D4a (OPN)	Pro-thrombin	Thrombin	SPARC	ATP	Thapsi-gargin
PC-3	+++	+++	+++	+++	++	+++	+++
DU-145	-	-	-	-	-	+++	++
LNCaP	-	-	-	-	-	-	+++
A10 (PCA2a)	-	-	-	-	-	-	+++
A11 (PCA2b)	-	-	-	-	-	-	+++

[a] All cell lines were cultured and loaded with fura-2 as described in Methods.

Figure legends:

Figure 1. Ca^{2+} signals in prostate cancer cell lines treated with bone fractions D3b and D4a. Panels A and C depict responses in PC-3 cells and panels B and D depict responses in DU-145 cells. All bone subfractions shown in Table 1 were tested for the ability to stimulate Ca^{2+} transients in both PC-3 and DU-145 cells. No bone fractions, other than those shown in this figure, including D2, which is enriched in α_2HS-glycoprotein, stimulated Ca^{2+} transients (data not shown). As seen, two bone fractions trigger an immediate Ca^{2+} signal in the PC-3 but not the DU-145 cells. Both cell lines respond to the subsequent addition of extracellular ATP (data not shown), indicating they can produce Ca^{2+} transients in response to external stimuli for which they express receptors. The vertical axis represents the intracellular Ca^{2+} concentration (nM).

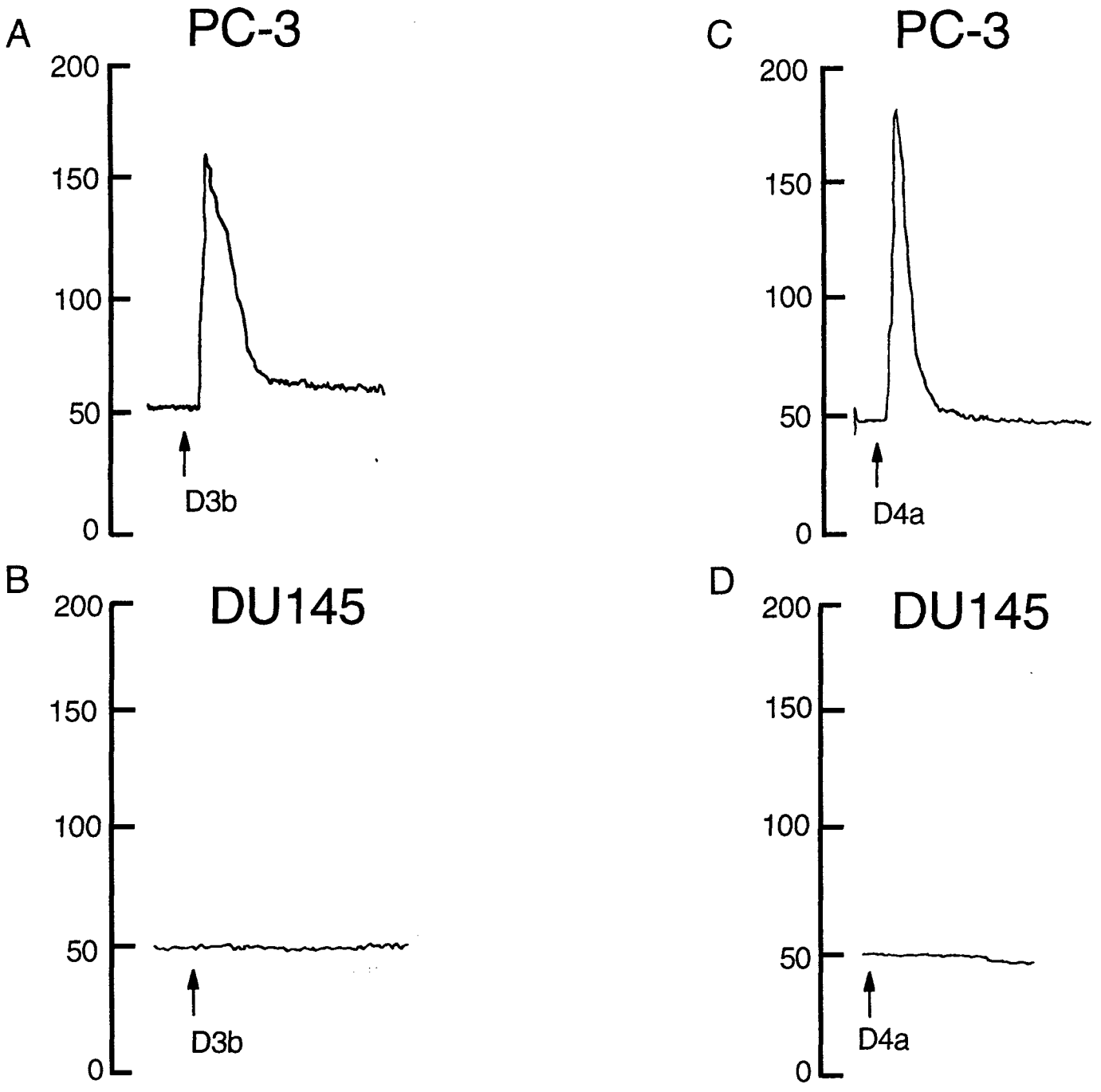
Figure 2. Ca^{2+} Oscillations in PC-3 and DU-145 Cells in Response to Extracellular ATP. Cell lines were loaded with fura-2, then Ca^{2+} transients measured in response to the addition of extracellular ATP (0.5 μM). Panel A. Development of Ca^{2+} oscillations in PC-3 cells; Panel B. Similar oscillations in DU-145 cells. As reported in Table 2, no such oscillations were produced by ATP addition to androgen-regulated LNCaP, A10 or A11 cells. The first arrow under the tracing denotes the addition of an inactive bone fraction; the second arrow denotes the time at which ATP was added.

Figure 3. Electrophoretic and sequencing analysis of proteins found in fraction D3b. The fraction D3b was separated into a series of individual protein bands by SDS-polyacrylamide gel electrophoresis as described in Methods. Following separation, bands were electroeluted and sequenced from the amino terminus. Although several bands demonstrated blocked amino termini (see text), the identity of several protein bands in this fraction was determined. Minor band 1, blocked; band 2, prothrombin; band 3, blocked; band 4, α_2HS serum glycoprotein fragment; band 4.5, SPARC; band 5, blocked.

Figure 4. Recording of Ca^{2+} transients in PC-3 cells treated with prothrombin and thrombin. Ca^{2+} signals in PC-3 cells in response to addition of prothrombin and thrombin were recorded following addition of the (pro)enzyme which was added at the points shown by the arrows. As seen, both proteins triggered a Ca^{2+} transient in PC-3 cells, suggesting that the activation of the thrombin receptor may be at least partially responsible for the Ca^{2+} activation seen with bone fraction D3b. Cell surface conversion of prothrombin to thrombin has been reported (see text).

Figure 5. Pseudocolor images in PC-3 cells treated with prothrombin. The series of panels shown in this figure correspond to the time-dependent Ca^{2+} signals in PC-3 cells treated with prothrombin. The time intervals shown correspond to time periods of 4 seconds, 1 minute 23 seconds, 1 minute 30 seconds, and 1 minute 42 seconds after addition of prothrombin. Red areas depict the highest Ca^{2+} concentrations (see Figure 4).

Figure 6. Ca^{2+} Signals In PC-3 Cells in Response to OPN2 and Blocked by Antibody LM609. Panel A. OPN2 isolated from ROS 17/2.8 cells (see text) produces a Ca^{2+} transient in PC-3 cells at concentrations between 5 and 10 μg/ml. The transient shown was produced by addition of 10 μg/ml OPN2. Panel B. Pre-treatment of fura-2 loaded PC-3 cells with antibody LM609 completely abolishes the Ca^{2+} response to addition of 10 μg/ml OPN2. This indicates a role for the $\alpha_v\beta_3$ integrin in the OPN-induced development of a Ca^{2+} transient in PC-3 cells.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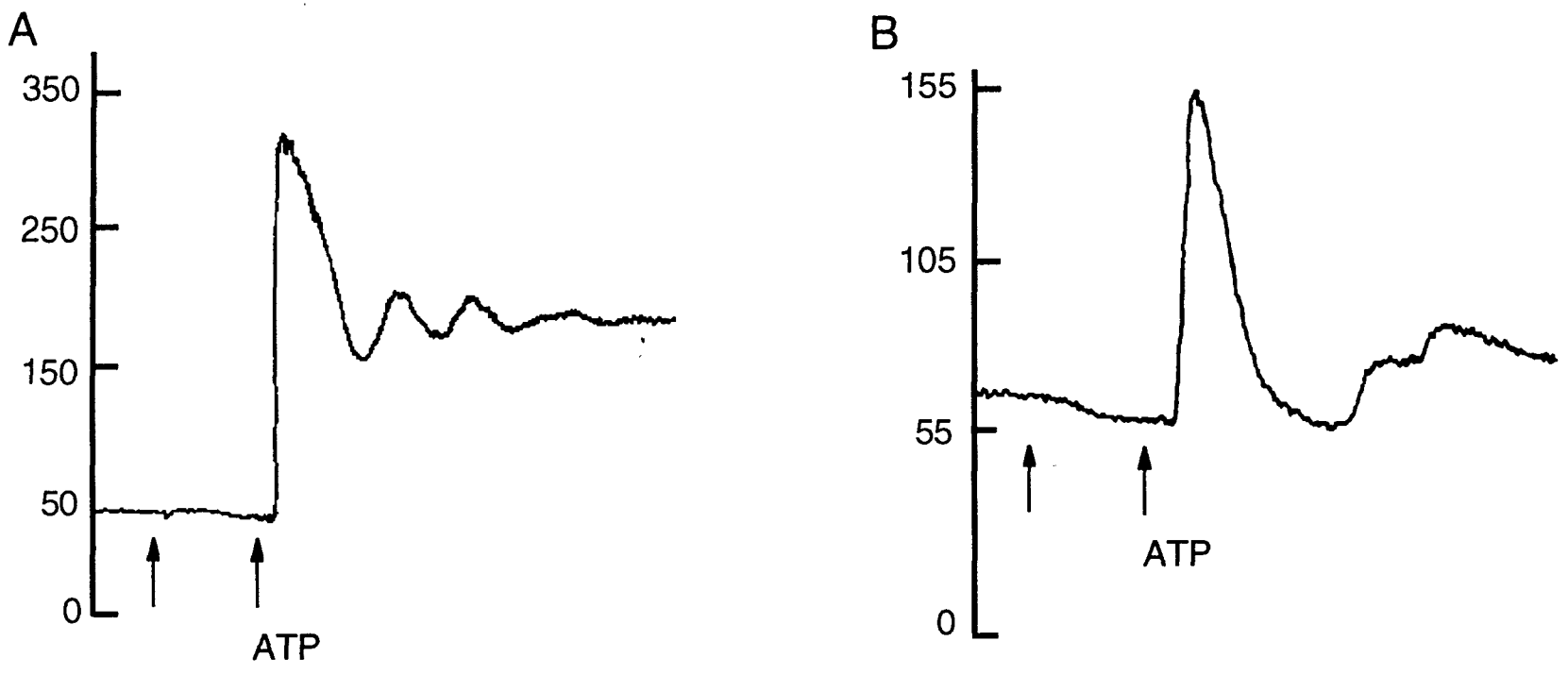
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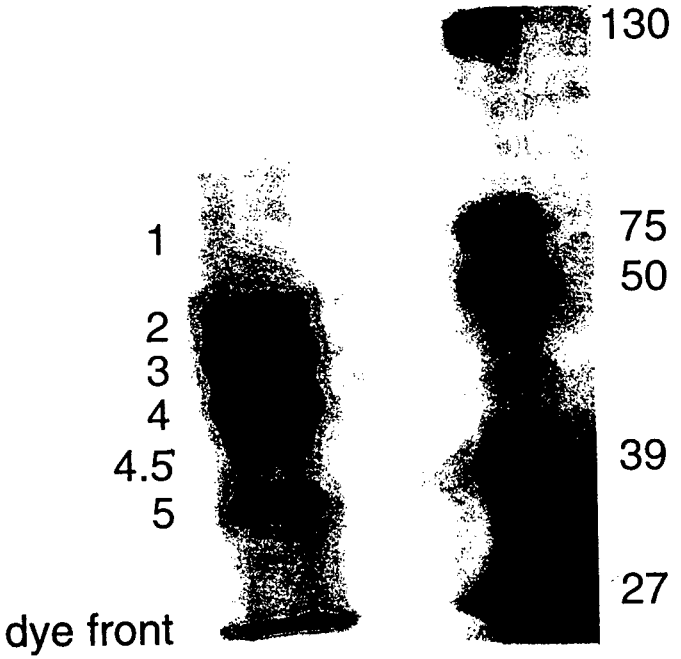
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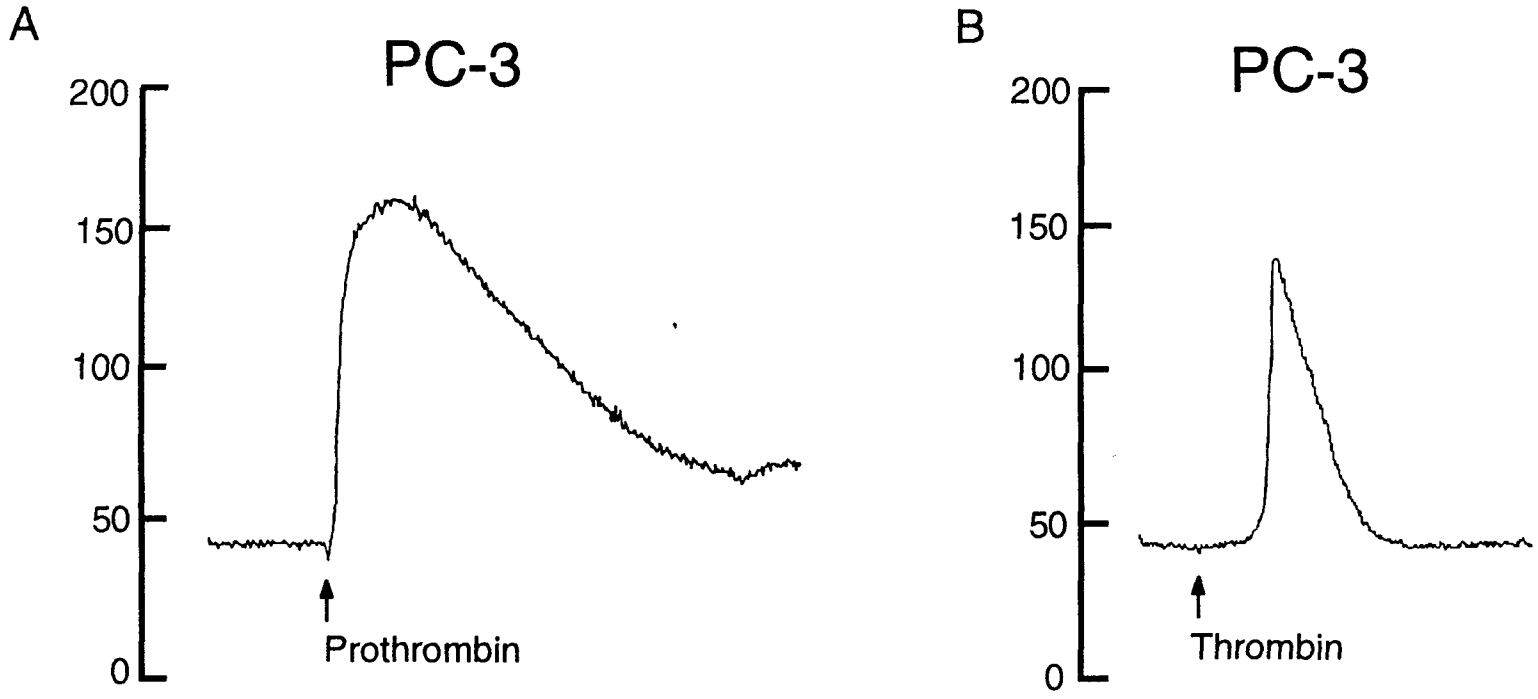
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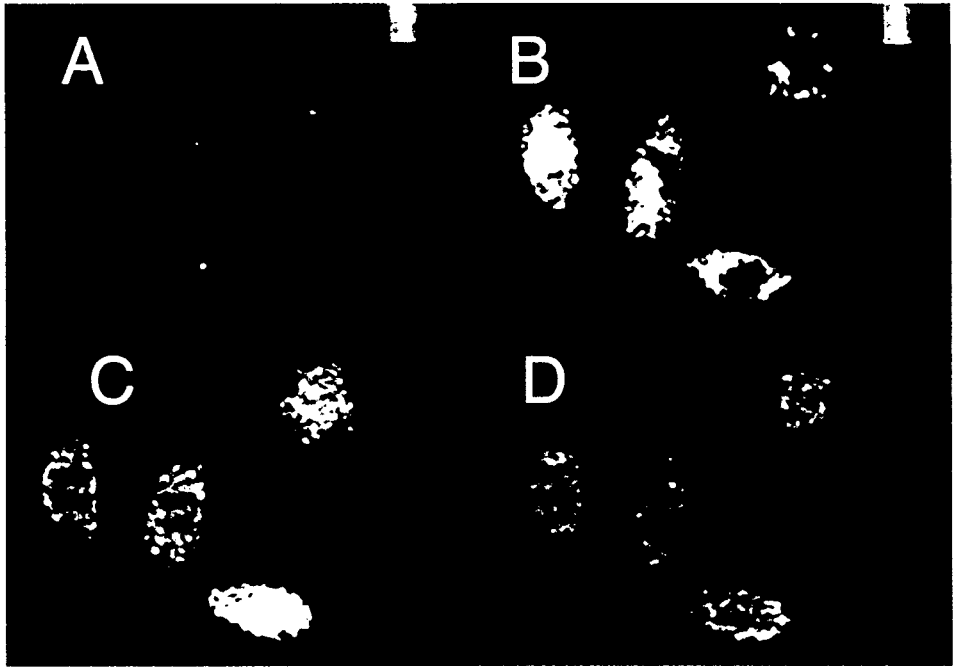
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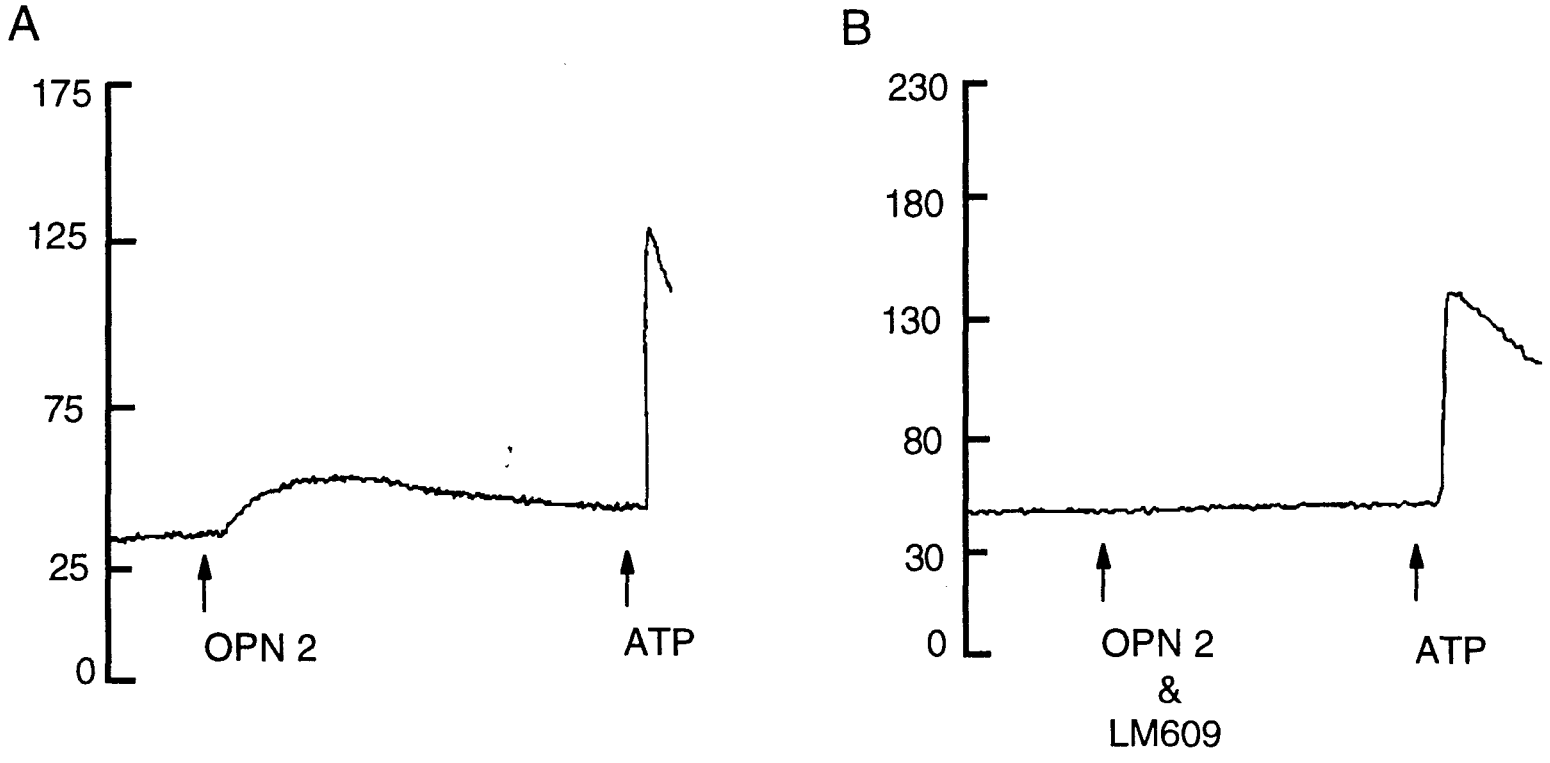
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DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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FORT DETRICK, MARYLAND 21702-5012

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ATTENTION OF:

MCMR-RMI-S (70-1y) 17 Jun 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for grants. Request the limited distribution statements for the Accession Documents listed at enclosure be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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